Microtubule stabilization specifies initial neuronal polarization

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Introduction

Neurons are highly polarized cells that typically have one thin, long process to transmit information (the axon) and several tapered, shorter processes to receive information (dendrites; Craig and Banker, 1994). One of the key questions of neurobiology is how a neuron acquires these polar structures, which provide the basis for unidirectional signal transmission. Previous studies focused on the actin cytoskeleton and its modulators, including nonactin regulating proteins that are involved in multiple processes, one common denominator appears to be their direct or indirect involvement in the control of microtubule (MT) dynamics. GSK-3β, for example, a multitarget protein kinase regulating many metabolic, signaling, and structural proteins (for review see Doble and Woodgett, 2003) is involved in the establishment and maintenance of neuronal polarity (Jiang et al., 2005; Yoshimura et al., 2005). Among its many functions, GSK-3β also modulates MT dynamics, e.g., by phosphorylating MT-associated proteins (MAPs; Goold et al., 1999; for review see Doble and Woodgett, 2003), whose binding to MTs is essential for neurite formation (Caceres and Kosik, 1990). It is noteworthy that some MAPs, including adenomatous polyposis coli protein, are inhibited by GSK-3β phosphorylation, whereas others, including MAP1B, are activated (Goold et al., 1999; for review see Doble and Woodgett, 2003). Consistent with the complex effects on MAPs, GSK-3β can either support or inhibit axonal growth depending on the extent of its inhibition (Kim et al., 2006). Overexpression of CRMP-2, another target of GSK-3β implicated in the regulation of MT dynamics and endocytosis, induces multiple axons in later developmental stages, which suggests a role for CRMP-2 in axon formation and maintenance (Inagaki et al., 2001). Another example is the SAD kinases, homologues of the conserved partitioning defective-1 (PAR-1) serine/threonine kinase, which acts in a variety of polarity events in species ranging from nematodes and flies to mammals (Wodarz, 2002).

Axonal growth and regeneration rely on the dynamic properties of the actin cytoskeleton and the microtubule framework. Key regulators of these intracellular processes include focal adhesion kinase (FAK), Crk, and CrkL, which are recruited to the nascent neurite tips and promote actin polymerization and microtubule dynamics (Witte et al., 2000). In this study, we addressed the role of MT dynamics in neuronal polarization and the mechanisms that control the onset of axon formation.

http://www.jcb.org/cgi/doi/10.1083/jcb.200707042

Supplemental material can be found at: http://doi.org/10.1083/jcb.200707042
SAD kinases are required for neuronal polarization (Kishi et al., 2005) and modulate presynaptic vesicle clustering (Crum et al., 2001) but also phosphorylate MAPs (Kishi et al., 2005). Recent work has also shown that activated c-Jun N-terminal kinase, so far known to be implicated in the regulation of gene transcription, cell death, and survival (for review see Bogoyevitch and Kobe, 2006), might play a role in axon formation (Oliva et al., 2006). c-Jun N-terminal kinase targets a wide variety of nuclear and cytoplasmic proteins, including transcription factors and actin-regulating proteins but also MAPs (for review see Bogoyevitch and Kobe, 2006).

In summary, despite the wealth of polarity regulators identified in the past years, our knowledge regarding the intracellular mechanisms that establish neuronal polarization has remained fragmentary. Given that some identified regulators of neuronal polarity appear to act indirectly or directly on MTs, the possibility arises that, in addition to the well-established function of the actin cytoskeleton, MTs may play a pivotal role in axon formation. The actual task of MTs in the establishment of neuronal polarity, however, has remained unclear and is still poorly understood. We therefore aimed to characterize whether MTs play an active role in neuronal polarization. Here, we find that the future axon has more stable MTs in its shaft and that stabilization of MTs is sufficient to induce axon formation. Our data show that MTs and the regulation of their stability play an instructive role in the initial polarization of neuronal cells.

Results

MT stability is increased in axons and one process of a subpopulation of morphologically unpolarized neurons

Earlier studies have yielded divergent results concerning the distribution of stabilized MTs in developing neuronal cells (Arregui et al., 1991; Dotti and Banker, 1991). We therefore first aimed to assess MT stability in developing axons and minor neurites using cultured rodent hippocampal neurons (Dotti et al., 1988). Upon plating, these cells form lamellipodia around the cell body (stage 1) that, after 12–24 h, condense to four to five processes, the minor neurites (stage 2). From this pool of morphologically indistinguishable processes, one starts to grow out quickly to become the axon (stage 3; Dotti et al., 1988). This first morphological sign of axon formation is a crucial hallmark of neuronal polarization, as it marks the initial break in symmetry during neuronal development (Craig and Banker, 1994).

As a first means to study MT stability, we extracted cells to remove unpolymerized tubulin subunits to assess MTs only and stained the cells for acetylated and tyrosinated α-tubulin, which are markers for stable and dynamic MTs, respectively (for review see Westermann and Weber, 2003). Acetylation of α-tubulin or enzymatic removal of its C-terminal tyrosine residue (detyrosination) gradually occurs in the MT polymer and is therefore found in long-lived, stable MTs, i.e., MTs with a low turnover that undergo few catastrophic events. In contrast, the presence of tyrosinated α-tubulin in MTs that has not yet undergone detyrosination denotes a recent assembly, i.e., tyrosinated α-tubulin is found in dynamic MTs with a high turnover. Expectedly, we found that tyrosinated MTs were predominant in the growth cones of all processes, which reflects their dynamic state required for steering and extension (Fig. 1, C and D; Tanaka et al., 1995). When we analyzed FRAP using neurons transfected with α-tubulin fused to GFP, we found that the GFP signal recovered faster in the growth cone compared with the axonal shaft. This further points to a high turnover of MTs in the growth cones (Video S1, available at http://www.jcb.org/cgi/content/full/jcb.200707042/DC1).

Morphologically polarized neurons (stage 3; Fig. 1 A) showed an enrichment of stable acetylated MTs in the axonal shaft compared with the shafts of minor neurites in 83.5 ± 1.0% of the cases (mean ± SEM, n = 709 neurons from three independent experiments; Fig. 1, B and D; and Fig. S1, A–D, available at http://www.jcb.org/cgi/content/full/jcb.200707042/DC1). On average from all stage 3 neurons, the axonal shaft showed a 3.2 ± 0.5-fold ratio increase of the fluorescence intensities of acetylated versus tyrosinated MTs compared with the shafts of minor neurites (P < 0.001 by t test; Fig. 1 B). Similar results were obtained by comparing acetylated to total α-tubulin integrated in MTs (Fig. S1, A–D and I).

Such differences in MT turnover already occur in morphologically unpolarized neurons (stage 2; Fig. 1 E). In 35.0 ± 6.1% of all stage 2 neurons, one neurite singled out and exhibited a significantly higher ratio of acetylated to tyrosinated MTs compared with the mean of the remaining neurites (P < 0.05 by Hampel outlier test; Fig. 1, F–H [arrowhead with asterisk], K [arrow], and M), which indicates that MT stabilization in one neurite precedes axon formation in morphologically still-unpolarized cells. Similar results were obtained by comparing acetylated to total MTs (Fig. S1, E–H and J). On average from all stage 2 neurons, the ratio of acetylated versus tyrosinated MTs was increased 1.9 ± 0.3-fold in the minor neurite with the highest ratio compared with the mean of the other neurites (P < 0.001 by t test; Fig. 1 J) and also significantly different from the neurite with the second-highest ratio alone (P < 0.001 by t test). The increased ratio was caused by a relative increase of acetylated over tyrosinated or total MTs within the process (Fig. 1, L and N; and Fig. S1, J–L). Thus, the axon of stage 3 neurons and one minor neurite of a subpopulation of stage 2 neurons show markers of lower MT turnover.

This differential distribution of posttranslational modifications also reflects an actual stability difference of MTs in axons and minor neurites. In 81.8 ± 5.9% of nodocazole short-term–treated polarized neurons (stage 3), MTs of minor neurites retracted more than axonal MTs (7.6 ± 0.9 and 11.9 ± 0.8 μm in axons and minor neurites, respectively; P < 0.001 by t test; Fig. 2, C, D, and I; and compare A and B). Similarly, morphologically unpolarized neurons (stage 2) showed a distinct difference between MT retraction in one neurite compared with the remaining neurites (3.1 ± 0.7 and 9.9 ± 1.0 μm, respectively; P < 0.001 by t test; Fig. 2, G, H, and J; and compare E and F), which suggests an early polarization of stabilized MTs during neuronal development. The MTs that resisted depolymerization were acetylated (Fig. 2, K, L, and N), whereas tyrosinated MTs had vanished after nocodazole treatment (Fig. 2, K, M, and N). Collectively, these data show that MT stability is increased in...
the axon of stage 3 neurons and one minor neurite of a subpopulation of stage 2 neurons.

**MT stability is changed in neurons whose polarity is affected by manipulation of GSK-3β or SAD kinases**

Next, we assessed whether molecules that regulate neuronal polarity affect MT stability in polarizing neurons. Neurons treated with the GSK-3β inhibitor SB415286 (10–20 μM), which has been reported to induce multiple axons per cell (Jiang et al., 2005; Yoshimura et al., 2005), showed an enrichment of stable, acetylated MTs in the supernumerary axons similar to wild-type axons (Fig. 3 A; compare with Figs. 1 D and 3 D). Notably, MT acetylation already increased upon GSK-3β inhibition in stage 2 neurons, which indicates an induction of MT stabilization by GSK-3β inhibition before axon formation (Fig. 3 B and C). For comparison to the multiaxonal phenotype induced by GSK-3β inhibition, we analyzed neurons from mice deficient in the PAR-1 homologues SAD A and B. These neurons show a polarity defect and lack a mature axon but generate multiple processes of similar lengths containing both the dendritic marker MAP2 as well as the axonal marker Tau-1 (Kishi et al., 2005). We found that SAD A/B kinase double knockout neurons lacked the specific enrichment of stable, acetylated MTs in a single process found in wild-type neurons as well as littermate control cultures (Fig. 3, E–G). Some of these neurons had neurites with MTs acetylated to a similar extent as in axons, whereas a majority had an acetylation level that rather resembled minor neurites. Despite this cell-to-cell variation (Fig. 3, E and F), however, acetylation levels were rather uniform within a given cell. The acetylation/tyrosination ratio of the longest versus the second longest neurite per cell was not significantly different in SAD A/B deficiencies (1.09 ± 0.03, P > 0.25 by t test), whereas control neurons showed a clear distinction (2.06 ± 0.05, P < 0.01 by t test; Fig. 3 H). Thus, specific alterations of neuronal polarity, including the formation of supernumerary axons or a loss of polarity, correlate with characteristic changes in MT stability.

**Moderate MT destabilization selectively reduces the formation of minor neurites**

SAD kinases and GSK-3β can act upstream of MT dynamics by controlling the affinity of MAPs (Jiang et al., 2005; Kishi et al.,...
The number of minor neurites, however, was significantly reduced in nocodazole-treated neurons (Fig. 4, C and D [arrowhead]) and minor neurites (B and F, arrowheads). In nocodazole-treated cells, MTs of minor neurites retract toward the cell body (D and H, arrowheads). MTs in the axon of stage 3 cells (D, arrow) and in one of the minor neurites of stage 2 cells (H, arrowhead with asterisk) are more resistant to depolymerization. Arrows, axons; arrowheads, minor neurites. (I and J) MT retraction after nocodazole treatment in polarized stage 3 (I) and morphologically unpolarized stage 2 (J) neurons (mean ± SEM; n = 119 and 50 neurons from five and three independent experiments, respectively; ***, P < 0.001 by t test). (K–N) Polarized rat hippocampal neurons with one axon (arrow) and several minor neurites (arrowheads) after 2 DIV before (K) and after (L–N) treatment with nocodazole (5 μM for 5 min). Tyrosinated (M and N, red) and acetylated (L and N, green) MTs were assessed. Bars, 20 μm.

2005; Yoshimura et al., 2005), which are known modulators of MT stability, yet they have various other cellular functions including the regulation of metabolism, signaling, gene transcription, and endocytosis (Crump et al., 2001; Grimes and Jope, 2001). We therefore wondered whether the observed differences in MT stability between axons and minor neurites play a direct role in polarization. We hypothesized that if MT stability is critical in distinguishing future axonal and dendritic fate, manipulations of MT dynamics should affect polarization. To test this assumption, we cultured neurons in the presence of low concentrations of the MT-destabilizing drug nocodazole, which moderately increase the catastrophe rate of MTs (15–75 nM; Vasquez et al., 1997). In control cells, the mean number of processes almost doubled from 1 (2.2 ± 0.2 processes per cell) to 3 d in vitro (DIV; 4.0 ± 0.1 processes per cell; P < 0.001 by analysis of variance [ANOVA]; Fig. 4 E), which reflects the formation of an axon and several minor neurites (Fig. 4, A and B). Cells grown in the presence of low concentrations of nocodazole were able to form and extend an axon likewise (Fig. 4, C and D, arrow). The number of minor neurites, however, was significantly reduced in nocodazole-treated neurons (Fig. 4, C and D [arrowhead], and E). This reduction was not caused by a general growth inhibition by nocodazole (Fig. 4 F) but by the development of many neurons with just one or two processes (Fig. 4, C–E). Expectedly, higher concentrations of nocodazole started to inhibit axonal outgrowth as well (Fig. 4 F).

Collectively, our data suggest that the future axon is able to overcome moderate MT-destabilizing conditions, whereas the outgrowth of minor neurites is impaired.

MT stabilization is sufficient to induce axon formation

To assess whether MT stabilization itself is sufficient to induce axon formation, we treated hippocampal neurons after 1 DIV with the MT-stabilizing drug taxol for varying times. Taxol-treated
blocked MT dynamics and axonal growth completely (unpublished data; Dehmelt et al., 2003). Taxol-induced processes also showed other typical axonal features. After 3 DIV, the number of cells with two or more processes positive for the axonal marker Tau-1 was increased fourfold in taxol-treated neurons compared with control neurons (Fig. 5, B, D, and F). At later stages of development, when axonal and dendritic proteins become segregated (Craig and Banker, 1994), the taxol-induced Tau-1-positive processes also showed the restriction of the dendritic marker MAP2 to the proximal part of the process as in control axons (Fig. 5, G–J, open arrowheads). In addition to the supernumerary axons, most of the cells had at least one or two dendrites. The taxol-induced processes clustered the presynaptic marker synapsin 1 later in development (10–12 DIV; Fig. 6), which was not observed in dendrites, further confirming neurons showed a drastic increase in neurite outgrowth (Fig. 5, A and E; and Fig. S2 A, available at http://www.jcb.org/cgi/content/full/jcb.200707042/DC1). The number of processes per cell exceeding 60 μm, a morphological characteristic of early axons, had increased more than twofold in the presence of low concentrations of taxol that favor MT polymerization (3–10 nM; Derry et al., 1995) for 2 d in comparison to control neurons (Fig. 5, A, C, and E). Taxol-induced processes showed a high ratio of acetylated to tyrosinated α-tubulin in MTs of unpolarized stage 2 neurons. The normalized average ratio of the five longest neurites is shown. Approximately 5 h after treatment with SB 415286, stage 2 neurons show a trend toward increased MT acetylation (23.0 ± 10.6%), which indicates a rise in MT stability. The increase is significant after ~8 h (47.4 ± 8.7%; mean ± SEM; n > 50 neurons per condition and time point from three independent experiments; *, P < 0.05 by t test). [D] Rat hippocampal neurons (3 DIV) treated with the GSK-3β inhibitor SB 415286 (10–20 μM treatment 6–8 h after plating) formed 2.1 ± 0.1 axons on average. These supernumerary axons show an increased ratio of acetylated to tyrosinated α-tubulin equal (P > 0.35 by t test) to that of the single axon of stage 3 control neurons (treatment with 0.04% DMSO; mean ± SEM; n > 35 neurons from three independent experiments). [E and F] Hippocampal neurons (3 DIV) derived from mice deficient for SAD A and B kinase show disturbed polarity and lack a defined axon. Instead, SAD A/B knockout neurons form multiple processes of similar length and uniform tubulin acetylation levels (see G), yet a high cell-to-cell variability. Bars, 20 μm. [G] Ratio quantification of fluorescence intensities of acetylated and tyrosinated α-tubulin in MTs. Processes of SAD A−/−B−/− neurons are short of the specific enrichment of acetylated MTs in one process found in wild-type as well as littermate control neurons (SAD A+/−B+/−; mean ± SEM; n = 66 and 27 neurons from three independent experiments for SAD A−/−B−/− and control, respectively). Note that the acetylation/tyrosination ratio varies slightly in control cells between species (rat vs. mouse; D and G). [H] Ratio of the acetylation/tyrosination ratios of the longest versus second longest process per cell for SAD A−/−B−/− and control neurons (mean ± SEM; ***, P < 0.001 by t test).
their axonal character. Our results therefore indicate that MT stabilization causes the formation of multiple axons.

Because MT acetylation was recently reported to promote kinesin-1–mediated cargo transport to specific neurites (Reed et al., 2006), we wondered whether the axon-inducing effect of taxol is related to its MT stabilizing function or the consecutive acetylation of MTs (Fig. S2 B). Neurons cultured in the presence of the deacetylase inhibitors tubacin (1 μM) and trichostatin A (TSA; 2 nM) featured a clear increase of MT acetylation in minor neurites similar to that of axons (Fig. S3, A–D, available at http://www.jcb.org/cgi/content/full/jcb.200707042/DC1). Polarity, however, was not altered in these neurons; they showed no increase in supernumerary axons compared with control neurons (Fig. S3 I) and the distribution of dephosphorylated Tau and MAP2 was indistinguishable from control cells (Fig. S3, E–H).

Thus, the axon-inducing effect of taxol appears to be linked to MT stabilization itself. Increasing acetylation of MTs does not per se affect neuronal polarity.

**Taxol application rapidly stabilizes MTs and shifts dynamic MTs to the tips of processes**

To assess MT dynamics during axon formation, polymerizing MT plus ends were visualized by transfecting neurons with the GFP-tagged MT plus end–binding protein 3 (EB3; Stepanova et al., 2003). Overall, the application of low concentrations of taxol (10 nM) reduced the dynamicity of MTs (Fig. S4, available at http://www.jcb.org/cgi/content/full/jcb.200707042/DC1). We observed a reduction of moving EB3-GFP particles in neurites to 51% of controls (≥500 moving EB3 particles of at least five neurons per condition quantified). Low concentrations of taxol, however, did not completely abolish MT dynamics like higher taxol concentrations used in earlier studies (unpublished data; Stepanova et al., 2003). Instead, low doses of taxol caused an accumulation of EB3-GFP at the tips of all minor neurites after taxol application in 84 ± 12% of the cases (Fig. 7 A–D; and Fig. S4). This directional shift toward the tip indicates the protrusion of polymerizing dynamic MTs to the growth cone periphery and was accompanied by neurite outgrowth (Fig. S4 and Video 1). Interestingly, in polarizing control neurons (Fig. 7, E and F), we observed in 64 ± 12% of the cases an enrichment of EB3-GFP in the presumptive future axonal growth cone, which was identified by its size and dynamics (Fig. 7, F and G, top, asterisk; Bradke and Dotti, 1997, 1999). Such enrichment was not seen in the growth cones of other neurites (Fig. 7 G).

We conclude that manipulations of MT stability cause drastic changes in neuronal polarity. Stabilizing MTs using taxol promotes polymerization at their plus ends, leading to an accumulation of dynamic MT plus ends at the neurite tips, which results in outgrowth of multiple axons.

**Local MT stabilization is sufficient to bias the site of axon formation**

During initial neuronal polarization, one out of several neurites is singled out to become the axon. It has been postulated that this selection is triggered by a positive feedback loop that allows sustained growth of the future axon (for reviews see Bradke and Dotti, 2000; Arimura and Kaibuchi, 2007). Hence, we analyzed whether MT stabilization could be part of such a proposed feedback loop by testing whether transient MT stabilization in one neurite is sufficient to trigger axon formation. One minor neurite of individual, unpolarized neurons (stage 2, 1 DIV) was
expected; P > 0.8 by χ² test; n = 28 neurons from nine independent experiments). The outcome of local uncaging in terms of site-directed axon formation was independent of the size of the growth cone and the length of the neurite receiving the UV pulses. Pulsed neurites could become the axon even if the neuron had other longer neurites with bigger growth cones that had a higher probability to form the axon (Fig. 8 A; Goslin and Banker, 1989; Bradke and Dotti, 1997, 1999), a phenomenon hardly observed in vehicle control experiments. Thus, a short trigger (10–15 min) of MT stabilization is already enough to bias the site of axon formation.

The immediate effects of local uncaging were visualized by performing the uncaging experiment with EB3-GFP–transfected neurons. Caged taxol was activated in a restricted area at the tip of a nongrowing process (Fig. 8, H and I, circle) with limited MT dynamics (visualized by the absence of EB3-GFP; Fig. 8 L, 1). Photoactivation of caged taxol promoted the protrusion of polymerizing MTs to the distal part of the process (Fig. 8, K and L, 2 and 3, arrow). This effect persisted after withdrawal of the trigger, i.e., after uncaging, and resulted in process outgrowth (Fig. 8, H, J, and L, 4 and 5). Local MT stabilization could also initiate outgrowth of a process when the cell already had another rapidly growing process (Fig. 8, H and J, arrowhead).
Axon formation correlates with increased MT stability, and stabilizing MTs is sufficient to induce axon formation. We show how molecules and pathways that act independent of actin dynamics can govern neuronal polarization by selective alteration of MT stability.

MT stability correlates with axon formation
Previous studies analyzing the MT network in developing neuronal cells had yielded differing results. One study found stable MTs just in the proximal part of the axon of cerebellar macro-neurons (Arregui et al., 1991) but another study could not confirm the confinement of stable acetylated MTs to the axon in hippocampal neurons (Dotti and Banker, 1991). By assessing the ratio of stable versus dynamic MTs in neuronal processes instead of individual posttranslational modifications of tubulin alone, we were able to reevaluate MT stability in developing neurons. We found that stable MTs are indeed not restricted to the axon but nevertheless predominate in the axonal shaft in comparison to minor neurites. The higher resistance of axonal MTs to depolymerization, which we observe, confirms increased MT stability in axons.
formed multiple axons with increased axon-like MT stability. High concentrations of taxol that hyperstabilize MTs and render them completely static, however, impede axon formation as previously described (unpublished data; Dehmelt et al., 2003), which indicates that a slight, balanced shift of MT dynamics toward more stable MTs is necessary to induce axon formation. Using EB3-GFP as a marker for polymerizing MTs in living neurons, we showed that stabilization enables MTs to polymerize more distally, resulting in net elongation of the axon.

Various pathways may act on MTs to promote MT stabilization in polarizing neurons. On the one hand, signaling pathways may stabilize MTs by regulating the affinity of MAPs to MTs and thereby changing their catastrophe rates, e.g., SAD kinases or GSK-3β/H9252. On the other hand, MT stabilization can also be achieved by a reduction of active depolymerization. For instance, overexpression of the Rac activator dedicator of cytokinesis 7 (DOCK7) induces multiple axons, which may be caused by DOCK7-mediated down-regulation of the MT-depolymerizing activity of stathmin (Watabe-Uchida et al., 2006). Unlike MAPs, stathmin acts on the plus ends of MTs yet its inactivation has the same outcome, a reduced catastrophe rate. CRMP-2, in turn, promotes MT assembly in the growing axon and induces multiple axons upon overexpression (Fukata et al., 2002). We propose that the fundamental process these pathways converge on is MT stabilization causes axon formation

When we analyzed neurons with altered polarity, we further substantiated the correlation that we had found between polarized MT stability and axonal identity. Neurons deficient in the PAR-1 homologues SAD A and B (Kishi et al., 2005) showed a loss of polarity that was accompanied by a loss of polarized MT stability. In contrast, when we induced multiple axons by inhibition of GSK-3β (Jiang et al., 2005; Yoshimura et al., 2005), we found that they exhibit a prevalence of stable MTs like normal axons. Among many other targets, SAD kinases and GSK-3β regulate the affinity of MAPs to MTs (Goold et al., 1999; Kishi et al., 2005), which, in turn, control MT dynamics. Moderate inhibition of GSK-3β, for example, reduces phosphorylation-dependent inactivation of specific MAPs (for review see Doble and Woodgett, 2003; Kim et al., 2006), leading to a stabilization of MTs by increased MAP binding. Our data show that polarization of MT stability and neuronal polarization are parallel events. Moreover, interfering with the regulation of MT stability disrupts proper establishment of neuronal polarity.

Figure 7. Taxol directs growing MT plus ends toward the tips of processes. (A–D) After 1 DIV, unpolarized neurons transfected with EB3-GFP (A) were subjected to low doses of taxol. The effect on MT dynamics was examined 30 min after treatment by monitoring the distribution of EB3-GFP (B–D). n = 52 neurons from eight independent experiments. (C) Higher magnification of the growth cones marked in A and B. EB3-GFP is mainly localized at the tips of neurites after a 30-min treatment with 10 nM taxol (a’–d’) in comparison to a more even distribution before treatment (a–d). (D) Profiles of EB3-GFP immunofluorescence intensity (arbitrary units) of a representative neurite (neurite “c”) before (red) and after (green) taxol treatment. (E–G) Dynamic MT plus ends in polarizing neurons (1 DIV) visualized by transfection with EB3-GFP. (G) Higher magnification of the growth cones marked in F. The growth cone of the future axon (F and G, asterisk) harbors a high amount of dynamic MTs in comparison to the growth cones of the remaining minor neurites in 64 ± 12% of the cases (n = 14 neurons from more than five independent experiments). Bars: (A and B) 20 μm; (C) 5 μm; (E and F) 20 μm; (G) 5 μm.
enhance neurite extension, thereby triggering maturation of the axon, including molecular and functional polarization. However, MT stabilization could act more directly on the polarization process itself.

**Increased MT stability in the axon that allows MTs to protrude with their dynamic ends more distally.**

Currently, it is still unclear how morphological and molecular polarization are linked. MT stabilization could on the one hand enhance neurite extension, thereby triggering maturation of the axon, including molecular and functional polarization. However, MT stabilization could act more directly on the polarization process itself.

**Figure 8. Local MT stabilization promotes axon formation.** (A and B) Rat hippocampal neuron (1 DIV) before (A) and after (B) UV-mediated photoactivation (circle) of caged taxol at the tip of one randomly chosen minor neurite. (C and D) Photoactivation did not interfere with the overall growth cone dynamics; most growth cones, including the pulsed one (arrow), are active. (E and F) 2 d after uncaging, the pulsed process had become the axon (arrow), which is Tau-1-positive (red) and MAP2-negative (green; F). (G) Probability of axon formation in the targeted area doubles after local activation of caged taxol compared with that expected by random chance (mean ± SEM; **, P < 0.01 by χ² test). Control treatment (DMSO and UV) does not influence randomized axon formation (P > 0.8 by χ² test). (H–L) Caged taxol was locally activated at the tip of a nongrowing neurite (circle) of an EB3-GFP-transfected neuron at 1 DIV. Before uncaging, the chosen neurite does not grow and shows little MT dynamics (H and I [arrow], and L, 1), whereas another neurite is rapidly growing (H and I, arrowhead; also see J and K). During uncaging ([L, 2 and 3]), the process becomes activated, visualized by enrichment of EB3-GFP at its tip ([L, 3, arrow]). After uncaging, the pulsed neurite shows increased thickness ([J and K]. Dynamic MTs keep protruding to the peripheral part of the process, promoting its outgrowth ([K and L, 4 and 5, arrow]. The asterisk in H and J indicates the initial position of the neurite tip in H. Bars: (A, B, and H–K) 20 μm; (C and D) 5 μm; (E and F) 50 μm; ([I] 10 μm.

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MT stabilization precedes axon formation

During initial neuronal development, one out of several seemingly equal neurites is singled out to become the axon. Our data suggest that MT stabilization in this neurite precedes morphological polarization based on the following observations: (a) MT stability is increased in one neurite of a subpopulation of morphologically unpolarized neurons; (b) neurons grown under mild MT-stabilizing conditions are able to form an axon, whereas formation of minor neurites is impaired, which suggests that only one minor neurite is able to overcome the destabilizing environment; and (c) local MT stabilization in one minor neurite by focal activation of a caged form of taxol strongly biases the site of axon formation. Consistent with our findings, the overexpressed kinesin-1 motor domain, which preferably binds to MTs containing markers of lower turnover (Reed et al., 2006), accumulates in the future axon before morphological polarization occurs (Jacobson et al., 2006). Our data argue that changes in MT dynamics are the primary cause of axon formation and that acetylation of MTs alone is not sufficient to induce initial axon formation.

How could local MT stabilization be achieved during axon formation? Presumably, either an environmental cue (Adler et al., 2006) or an internal signal like centrosome localization (Zmuda and Rivas, 1998; de Anda et al., 2005) could initiate a local imbalance inside the MT network. In the nematode Caenorhabditis elegans, for example, directional netrin cues define the site of axon initiation of a subtype of motor neurons by promoting a phosphoinositide 3-kinase (PI3K) signaling-dependent asymmetrical distribution of the actin regulator MIG-10/lamellipodin (Adler et al., 2006). Interestingly, asymmetrical PI3K signaling itself plays an important role in neuronal polarization (Menager et al., 2004) and promotes MT stabilization in migrating fibroblasts (Onishi et al., 2007). Thus, netrin-induced axon formation may function, at least in part, via regulation of MT dynamics, which is in line with the netrin turning response depending on MTs (Buck and Zheng, 2002) and MAP1B (Del Rio et al., 2004). Similarly, local inactivation of GSK-3β in one neurite of an unpolarized neuron may induce axon outgrowth by MAP-mediated MT stabilization (Kim et al., 2006). Interestingly, the small GTPase R-Ras, which can inhibit GSK-3β via PI3K and Akt-kinase, is selectively localized to a single neurite of unpolarized neurons (Oinuma et al., 2007) and might therefore account for local GSK-3β inactivation.

During early developmental stages, the centrosome and the Golgi apparatus cluster close to the area where neurites form their first neurite, which will later become the axon (Zmuda and Rivas, 1998; de Anda et al., 2005). The localization of the centrosome may therefore be responsible for an early bias of MT stability between different neurites. As the centrosome position is controlled by PI3K and Cdc42 (Etienne-Manneville and Hall, 2003), however, it may not be the cause for local MT stabilization but rather a result of axon-inducing signaling (for review see Arimura and Kaibuchi, 2007). Alternatively, a bias could come from CLIP-associated protein (CLASP)–mediated nucleation of noncentrosomal MTs at the Golgi network (Efimov et al., 2007) or other localized MT-stabilizing factors including EB1 or EB3, adenomatous polyposis coli protein, and mDia (Nakagawa et al., 2000; Wen et al., 2004; Votin et al., 2005).

Interestingly, the local MT stabilization in morphologically unpolarized neurons, which we observe, also offers an explanation for the increased membrane traffic that precedes axon formation (Bradke and Dotti, 1997). MT-dependent motor proteins show a higher affinity toward stabilized MTs (Liao and Gundersen, 1998; Reed et al., 2006), which is in line with enhanced vesicle transport on stable MTs (Lin et al., 2002; Nakata and Hirokawa, 2003). Increased MT stability in the future axon may therefore lead to polarized membrane flow and contribute to determining the site of axon formation.

In summary, we present here for the first time direct evidence that MT stability is an active determinant of neuronal polarization. Recent work has shown that actin and MTs mutually influence each other (Basu and Chang, 2007). Taking into account our findings about the instructive role of MTs in neuronal polarization, we hypothesize that the initial trigger for axon formation could derive from both actin and MTs. Their reciprocal regulation in turn may drive a positive feedback loop that sustains axonal growth. The next challenge will be to characterize this molecular interplay of MTs and the actin cytoskeleton during neuronal polarization. Moreover, molecules involved in the regulation of MT dynamics in processes like cell migration or growth cone steering should be reassessed for a potential role in neuronal polarization. Interestingly, we recently showed that MT organization is a crucial factor for the formation of the two distinct structures after axonal lesioning: actively protruding growth cones and nongrowing retraction bulbs (Ertürk et al., 2007). We therefore believe that the ability of taxol to convert a nongrowing minor neurite into a growing axon should be explored in situations where process growth is restrained, e.g., after central nervous system lesioning.

Materials and methods

Cell culture

Primary hippocampal neurons derived from rat embryos were cultured as described previously (de Hoop et al., 1997). In brief, the hippocampi of embryonic day (E) 18 rats or mice were dissected, trypsinized (0.05% Trypsin-EDTA; Invitrogen) and washed in HBSS. Cells were then dissociated with glass Pasteur pipettes and 1–1.3 × 10^5 cells were plated onto poly-lysine-coated glass coverslips in 6-cm Petri dishes containing MEM and 10% heat-inactivated horse serum. The cells were kept in 5% CO_2 at 36.5°C. After 6–12 h, the coverslips were transferred to a 6-cm dish containing astrocytes in MEM and N2 supplements. Hippocampal neurons from E18 SAD A SAD B knockout mice (provided by J. Sanes, Harvard Medical School, Boston, MA) were cultured as described previously using wild-type neuronal cultures. For mixed wild-type/GFP neuronal cultures, we combined wild-type mouse hippocampal neurons with 1–3% of hippocampal neurons isolated from mice expressing EGFP (provided by M. Götz, German Research Center for Environmental Health, Neuberberg, Germany; Okabe et al., 1997).

Video microscopy and image acquisition

Living neurons were kept at 36°C on the stage of a microscope (Axiovert 135TV; Carl Zeiss, Inc.). Glass-bottom dishes (Mettek Corporation) filled with Hapes-buffered HBSS were used for observation with LD A-Plan 32× NA 0.4 or Plan Apochromat 40× NA 1.0 objectives (both from Carl Zeiss, Germany). Alternatively, custom-made cell chambers served for live observation with LD A-Plan 32× NA 0.4 or Plan Apochromat 40× NA 1.0 objectives (both from Carl Zeiss, Germany). After central nervous system lesioning.

When necessary, drugs were diluted to 4× concentration in HBSS and added on stage during the observation. Cells were illuminated with 100 W, 12 V halogen light or fluorescence light from a 103W/2 mercury short arc bulb. Halogen light was set to minimal intensity to avoid phototoxicity and fluorescence radiation was reduced to 5 or 25% with transmission filters to avoid phototoxicity and reduce bleaching.
Images were captured using a camera (4912 series; Cohu) at room temperature (fixed samples) or 36 °C (living neurons). The camera was connected to a charge-coupled device camera control panel (C 2741; Hamamatsu). Pictures were recorded using an image grabber (LG3) and Scion Image Beta 4.0.2 (both from Scion Corp.).

FRAP
FRAP experiments were performed with a live cell imaging setup (Delta Vision Rt; Applied Precision) based on an inverted fluorescence microscope (IX71; Olympus) with a UPlanApo 100× NA 1.35 objective (Olympus). The setup included a quantifiable laser module (488-nm diode laser) for bleaching GFP and an incubation chamber (Solent Scientific). Living neurons were imaged in Hepes-buffered HBSS at 36 °C. Images were acquired using a camera (Photon Technologies CoolSnap HQ; Roper Scientific). SoftWoRx 3.5.0 (Applied Precision) was used for image recording, deconvolution (based on measured point spread function and the additive iteration method), and analysis of the FRAP experiments.

Transfection and protein expression
Neurons were transfected before plating with the Amaza Nucleofector system using highly purified DNA (EndoFree Maxi Prep; Qiagen). Directly after isolation of hippocampal neurons, 3 μg of pEGFP-N1-EB3 or 7.5 μg of pEGFP-T7/C1-α-tubulin plasmid DNA was used for electroporation of 500,000 cells according to the manufacturer’s instructions. Subsequently, neurons were plated in 6-cm dishes containing MEM and 10% heat-inactivated horse serum and further cultured as described in Cell culture. pEGFP N1 EB3 was provided by A. Akhmanova (Erasmus Medical Center, Rotterdam, Netherlands) and V. Small (Institute of Molecular Biotechnology of the Austrian Academy of Sciences, Vienna, Austria). pEGFP-T7/C1-α-tubulin was a gift from E. Nigg and S. Nagel (Max Planck Institute of Biochemistry, Martinsried, Germany).

Immunocytochemistry
To stain for Tau-1, MAP2, GFP, or synapsin, cells were fixed in 4% paraformaldehyde, quenched in 50 mM ammonium chloride, and permeabilized with 0.1% Triton X-100 for 3–5 min. To assess acetylated, tyrosinated, and total tubulin integrated in MTs without unpolymerized tubulin subunits, cells were simultaneously fixed and permeabilized in PHEM buffer (60 mM Pipes, 25 mM Hepes, 5 mM EGTA, and 1 mM MgCl2) containing 0.25% glutaraldehyde, 3.7% formaldehyde, 3.7% sucrose, and 0.1% Triton X-100 (adapted from Smith, 1994) and quenched as above. The neurons were then blocked at room temperature for 1 h in a solution containing 2% fetal bovine serum (Invitrogen), 2% bovine serum albumin (Sigma-Aldrich), and 0.2% fish gelatin (Sigma-Aldrich) dissolved in phosphate-buffered saline. Subsequently, cells were incubated with primary antibodies diluted in 10% blocking solution. The primary antibodies used were: Tau-1 (1:5,000; Millipore), anti-MAP2 (1:6,000; Millipore), anti-α-tubulin (clone B-5-1-2, 1:20,000; Sigma-Aldrich), anti-tubulin (rabbit polyclonal, 1:200; Sigma-Aldrich), anti-acetylated tubulin (clone 6-11B-1, 1:500; Sigma-Aldrich), anti-tubulin (clone Y181/2, 1:40,000; Abcam), anti-GFP (1:15,000; United States Biological), or anti-synapsin 1 (1:200; Millipore).

For visualization of F-actin, 4 U/ml rhodamine-coupled phalloidin (stored as a 200 U/ml methanol stock solution at −20 °C) was used (Invitrogen).

As secondary antibodies, Alexa Fluor 488, 550, or 568-conjugated goat anti-mouse, anti-rabbit, or anti-rat IgG antibodies or Alexa Fluor 488 or 555-conjugated donkey anti-mouse or anti-goat (all 1:50; Invitrogen) were used.

Drug treatment
Depending on the experiment, 0.3–100 nM taxol (Sigma-Aldrich), 1 μM cytochalasin D (Sigma-Aldrich), 15–225 nM nocodazole (Sigma-Aldrich), 10–20 μM SB415286 (Tocris Bioscience), 2 nM TSA (Cell Signaling Technology), and 1 μM tubacin were added to culture medium 6–24 h after plating and cells were further incubated at 36.5 °C in the presence of the drug. Drugs were kept as stock solutions in DMSO (5 mM taxol, 10 mM cytochalasin D, 6.67 mM nocodazole, 25 μM SB415286, or 20 mM tubacin) or ethanol (4 mM TSA) at −20 °C. Tubacin was provided by R. Mazitschek and S. Schreiber (Harvard University and Massachusetts Institute of Technology, Cambridge, MA).

To assess MT stability, neurons grown on a coverslip with a relocation grid (Laser Zentrum Hannover) were transferred to Hepes-buffered HBSS, located, and imaged. The cells were subsequently treated with 0.075% DMSO or nocodazole (3.3–5 μM) for 5 min and then simultaneously extracted and fixed to remove unpolymerized tubulin subunits and allow clear visualization of MTs. F-actin outlining the neuron was used as a reference point to measure the retraction of MTs from the distal end of the processes toward the cell body after partial depolymerization of the MT cytoskeleton.

Image analysis and quantification
Length and intensity measurements were performed using Scion Image Beta 4.0.2 for Microsoft Windows or ImageJ analysis software. Plot profiles of fluorescence intensity were created with ImageJ. Average neurite length in experiments with long term nocodazole treatment was determined taking into account neurons without processes. To analyze movement of fluorescent particles in EB3-GFP-transfected neurons, both phase and fluorescent images were acquired in 5–10 s intervals during the experiment. Subsequently, kymographs from the regions of interest were made from the individual images using Scion Image and a purpose-written macro. EB3-particles were only considered for analysis if they could be followed clearly for three or more frames in the kymographs. The ratio of acetylated versus tyrosinated α-tubulin was determined from the mean fluorescence intensity of both channels in a square of 3×3 to 5×5 pixels in the medial part of each process after background subtraction with Photoshop (Adobe).

Focal photoactivation of caged taxol
Near-UV light (λ = 365 nm) was focused on a spot of 20–30 μm in diameter using the fluorescence iris of a microscope (Axiovert 135 TV) equipped with a Plan Apochromat 40× oil immersion objective (both from Carl Zeiss, Inc.). For the uncaging experiment with EB3-GFP-transfected neurons, a Plan Apochromat 63× oil immersion objective (Carl Zeiss, Inc.) was used instead and the cells were handled as described in Video microscopy and copy image acquisition. Under phase light, one dynamic growth cone of an unpolarized stage 2 neuron grown on a relocation coverslip was randomly chosen. Subsequently, caged taxol or DMSO was added to the neurons on the microscope stage to a final concentration of 1–10 nM or 0.02%, respectively. After equilibration, the tip of the selected growth cone or the region directly adjacent to it was pulsed for 10–15 min with near-UV light (pulse duration 50–100 ms; frequency 0.2–0.33 Hz). After uncaging, the neurons were incubated for another 2 d in the incubator.

Neurons were then fixed, stained for Tau-1 and MAP2, and relocated, and the site of axon formation was determined. Only neurons that had formed an axon during the 48 h after uncaging were taken into account. For analysis, we measured the angle between the edge of the uncaged area, the center of the cell body, and the site of axon formation.

For calculation of the probability of random axon formation, the number of growth cones within the sector in question was divided by the total number of the cells’ growth cones.

Online supplemental material
Fig. S1 shows the distribution of acetylated versus total tubulin in hippocampal neurons. Fig. S2 illustrates that deacetylation of axon-like processes with increased MT stability by taxol. Fig. S3 shows that deacetylation of MTs alone is not sufficient to induce axon formation. Fig. S4 shows that taxol directs growing MT plus ends toward the tips of the processes. Fig. S5 illustrates an increase of MT acetylation in the pulsed neurite after local uncaging of photoactivatable taxol. Video 1 demonstrates a high MT turnover in neuronal growth cones by assessing FRAP in α-tubulin-GFP-transfected neurons. Video 2 visualizes MT dynamics in EB3-GFP-transfected neurons upon taxol addition. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200707042/DCC.

We are deeply indebted to Ms. Liane Meyn for preparing hippocampal neurons. We would like to thank Drs. Axel Boldt, Cord Brokhausen, Reinhard Fassler, Boyan Garvalov, Farida Hellal, Rüdiger Klein, Thomas Mayer, Gaia Tavosanis, Hans Thoenen, and Bhavana Yera for discussing the manuscript. We are also deeply indebted to Dr. Magdalena Götz for providing transgenic GFP mice, Dr. Joshua Sanes for providing SAD A/B knockout mice, and Drs. Ralph Mazitschek and Stuart Schreiber, who provided us with tubacin through the Initiative for Chemical Genetics at the National Cancer Institute. We would also like to thank Anna Akhmanova for donating Tocris Bioscience, Reinhard Fassler, Boyan Garvalov, Farida Hellal, Rüdiger Klein, Thomas Mayer, Gaia Tavosanis, Hans Thoenen, and Bhavana Yera for discussing the manuscript. We are also deeply indebted to Dr. Magdalena Götz for providing transgenic GFP mice, Dr. Joshua Sanes for providing SAD A/B knockout mice, and Drs. Ralph Mazitschek and Stuart Schreiber, who provided us with tubacin through the Initiative for Chemical Genetics at the National Cancer Institute. We would also like to thank Dr. Magdalena Götz for providing transgenic GFP mice, Dr. Joshua Sanes for providing SAD A/B knockout mice, and Drs. Ralph Mazitschek and Stuart Schreiber, who provided us with tubacin through the Initiative for Chemical Genetics at the National Cancer Institute. We would also like to thank Dr. Magdalena Götz for providing transgenic GFP mice, Dr. Joshua Sanes for providing SAD A/B knockout mice, and Drs. Ralph Mazitschek and Stuart Schreiber, who provided us with tubacin through the Initiative for Chemical Genetics at the National Cancer Institute. We would also like to thank Dr. Magdalena Götz for providing transgenic GFP mice, Dr. Joshua Sanes for providing SAD A/B knockout mice, and Drs. Ralph Mazitschek and Stuart Schreiber, who provided us with tubacin through the Initiative for Chemical Genetics at the National Cancer Institute. We would also like to thank Dr. Magdalena Götz for providing transgenic GFP mice, Dr. Joshua Sanes for providing SAD A/B knockout mice, and Drs. Ralph Mazitschek and Stuart Schreiber, who provided us with tubacin through the Initiative for Chemical Genetics at the National Cancer Institute.


